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Article

The Sirtuins Hst3 and Hst4p Preserve Genome Integrity by Controlling Histone H3 Lysine 56 Deacetylation

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Summary

Background: Acetylation of histone H3 lysine 56 (K56Ac) occurs transiently in newly synthesized H3 during passage through S phase and is removed in G2. However, the physiologic roles and effectors of K56Ac turnover are unknown.

Results: The sirtuins Hst3p and, to a lesser extent, Hst4p maintain low levels of K56Ac outside of S phase. In *hst3 hst4* mutants, K56 hyperacetylation nears 100%. Residues corresponding to the nicotinamide binding pocket of Sir2p are essential for Hst3p function, and H3 K56 deacetylation is inhibited by nicotinamide in vivo. Rapid inactivation of Hst3/Hst4p prior to S phase elevates K56Ac to 50% in G2, suggesting that K56-acetylated nucleosomes are assembled genome-wide during replication. Inducible expression of Hst3p in G1 or G2 triggers deacetylation of mature chromatin. Cells lacking Hst3/Hst4p exhibit many phenotypes: spontaneous DNA damage, chromosome loss, thermosensitivity, and acute sensitivity to genotoxic agents. These phenotypes are suppressed by mutation of histone H3 K56 into a nonacetylatable residue or by loss of K56Ac in cells lacking the histone chaperone Asf1.

Conclusions: Our results underscore the critical importance of Hst3/Hst4p in controlling histone H3 K56Ac and thereby maintaining chromosome integrity.

Introduction

Newly synthesized histones deposited genome-wide during replication are acetylated at several lysine residues within their amino-terminal tails [1–4]. Recent work with *Saccharomyces cerevisiae* has established that specific residues within the nucleosome core are also acetylated. These include lysine 56 of H3 [5–10] and lysine 91 in H4 [11]. In higher eukaryotes, acetyl groups of newly synthesized histones are rapidly removed [1, 2, 12]. No comparable study has been performed in yeast. However, bulk histone acetylation is rapidly turned over in *S. cerevisiae* [13, 14]. K56Ac of new histones deposited in S phase is normally removed before mitosis, but the function of this turnover is not known.

Sirtuins are evolutionarily conserved proteins related to *S. cerevisiae* Sir2p, an NAD^+-dependent histone deacetylase (HDAC) active on N termini of H3 and H4 [15–19]. The *S. cerevisiae* genome encodes four additional sirtuins, encoded by the *HST1* (Homologous to Sir Two) to *HST4* genes [20], while the human genome encodes at least seven sirtuins [21, 22]. Some sirtuins act on nonhistone proteins. *Salmonella enterica* CobB and human SIRT2 deacetylate acetyl-coenzyme A synthetase and tubulin, respectively [23, 24]. Among the yeast sirtuins, Hst3 and Hst4p are closely related to each other and more distantly related to Sir2p [20]. Single mutants in *hst3* and *hst4* show mild phenotypes, but *hst3 hst4* cells exhibit pronounced defects in cell-cycle progression, mitotic chromosome transmission, DNA damage susceptibility, and thermosensitivity [20], suggesting considerable functional overlap between these sirtuins. However, the substrate(s) of these two sirtuins have remained elusive.

Here, we demonstrate a role for Hst3 and Hst4p in cell-cycle regulation of H3 K56Ac. The entire genome is completely and permanently K56 acetylated in *hst3 hst4* mutants. Constitutive K56Ac underlies the *hst3 hst4* phenotypes, which are significantly suppressed by point mutation of K56 to arginine (K56R). Our experiments argue that deacetylation of K56Ac is crucial to preserving genome integrity.

Results

Hst3 and Hst4p Control K56Ac during the Cell Cycle

To identify proteins regulating K56Ac during the cell cycle, we tested yeast strains lacking multiple known HDAC enzymes by immunoblotting with K56Ac-specific antibodies [6]. K56Ac normally occurs transiently during S phase [6]. G2/M-arrested yeast strains lacking several class I and II HDACs had normal K56Ac levels (Figure 1A), whereas a strain lacking all five sirtuins had strikingly elevated levels of K56Ac. Among the sirtuin single mutants, only *hst3* exhibited elevated K56Ac levels and these were exacerbated in *hst3 hst4* cells (Figures 1B and 1C). Except for *hst4*, none of the other sirtuin mutations increased K56Ac in *hst3* cells. Cell-cycle analysis showed that wild-type cells, *hst3* and *hst4* single mutants had no detectable K56Ac in G1 (Figure 1C). In contrast, K56Ac in *hst3 hst4* mutants was

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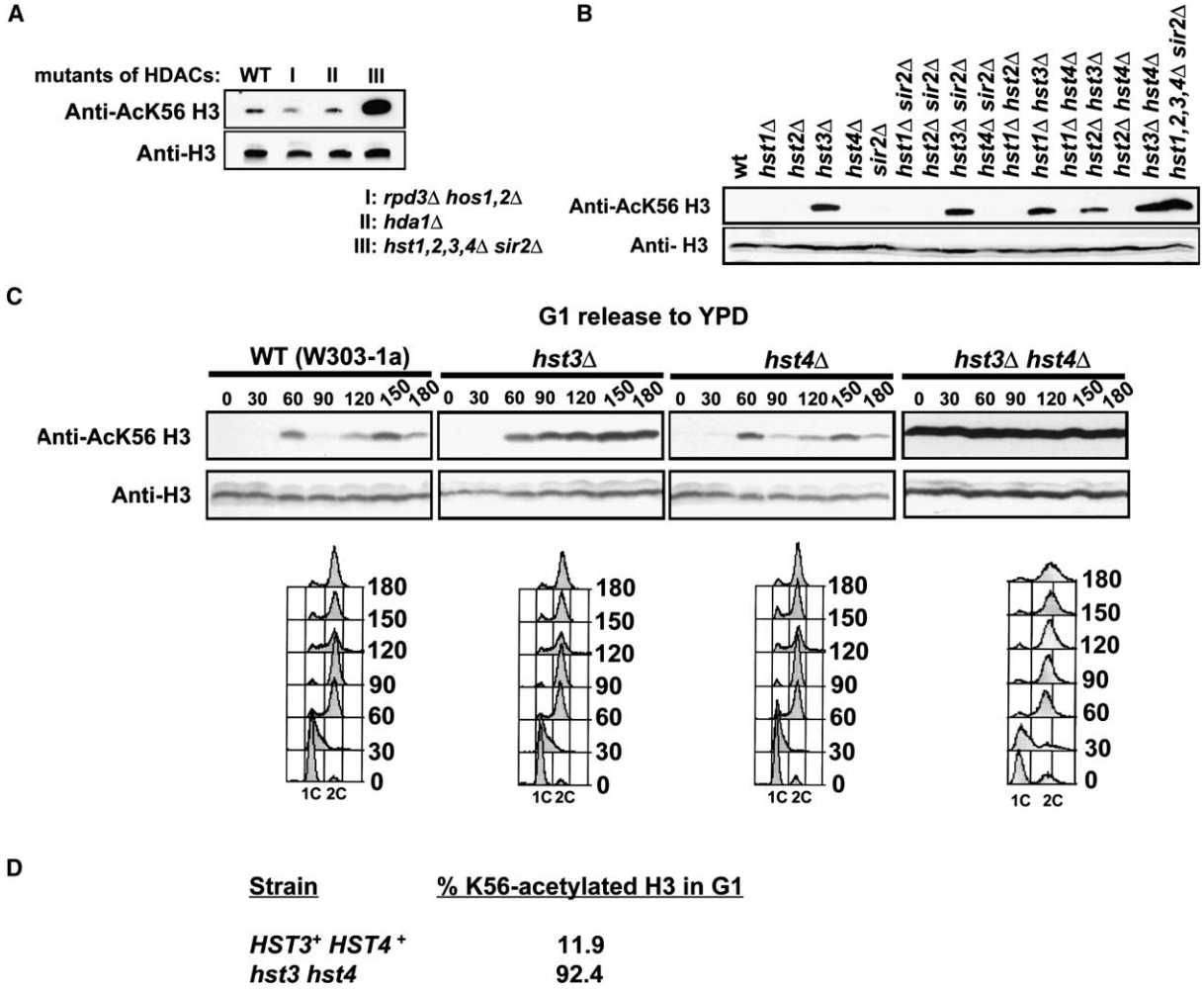

Figure 1. Hst3/Hst4p Control K56Ac during the Cell Cycle

(A and B) Yeast strains were arrested in G2/M phase with nocodazole. Immunoblots were probed for K56Ac or total H3.

(C) Yeast strains were synchronized in G1 phase with α-factor and released into the cell cycle, and immunoblots were probed for K56Ac or total H3. Cell-cycle progression was monitored by flow cytometry. Times (min) after G1 release are shown.

(D) The proportion of K56Ac H3 was quantified by mass spectrometry with histones purified from wild-type and *hst3 hst4* mutant cells arrested in G1 phase with α-factor.

present in G1 and did not fluctuate during the cell cycle (Figure 1C). Based on quantitative mass spectrometry, essentially all H3 molecules were K56 acetylated in *hst3 hst4* double mutants (Figure 1D), explaining why acetylation was not cell cycle regulated. One notable difference between the two single mutants was that *hst3* mutants retained high levels of K56Ac in G2/M, whereas most K56 acetylation disappeared in *hst4* single mutants and wild-type cells (Figure 1C). These observations agree with observed peaks of *HST3* mRNA and protein in G2/M ([25]; data not shown). Additionally, these results suggest that constitutive hyperacetylation could underlie *hst3 hst4* phenotypes (see below).

The Hst3p Nicotinamide Binding Pocket Is Required for K56Ac Regulation

The activity of Sir2p is inhibited by nicotinamide [26]. In the continuous presence of 25mM nicotinamide, K56Ac steadily increased over a 6 hr period that encompassed several cell divisions (Figure 2A, see Figure S1 in the Supplemental Data available with this article online). Consistent with K56Ac deacetylation by a sirtuin, nicotinamide inhibited deacetylation of newly synthesized histones during passage through S phase (Figure S6). In contrast, nicotinic acid did not increase K56Ac (Figure S1). Nicotinamide also sensitized wild-type cells to genotoxic agents, phenocopying *hst3 hst4* mutants (data not shown).

Hst3 and Hst4p are related to the catalytic core of the NAD^+-dependent deacetylase Sir2p (Figure 2B). We made mutations in three invariant residues of the Hst3p core and examined their effects in vivo. Two residues, N152 and D154, lie within the C-pocket, a highly conserved nicotinamide binding region in other sirtuins [27, 28]. The third residue changed was the conserved histidine, H184. This invariant histidine has been proposed to act as a general base during catalysis [29, 30]. Mutation of this residue interferes with Sir2p deacetylase activity and function in vivo [15, 27, 31], although equivalent mutations in Hst2p and in TmSir2 reduce but

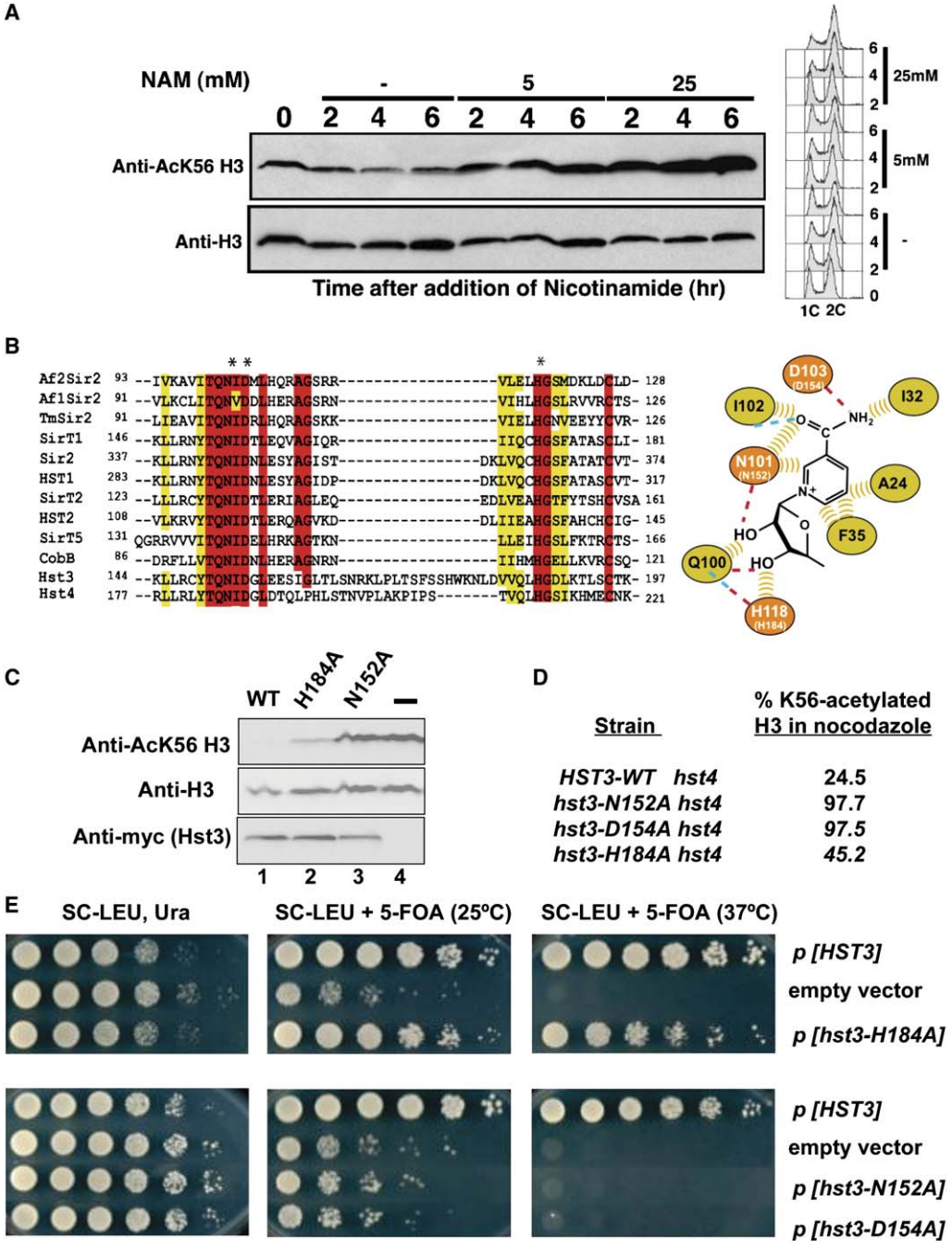

Figure 2. Nicotinamide and Hst3 Mutations Increase K56Ac

(A) Nicotinamide (NAM) was added to asynchronously growing wild-type cells (W303 *MAT***a**) at the indicated concentrations for up to 6 hr. Immunoblots were probed for K56Ac or total H3. Cell-cycle progression was monitored by flow cytometry.

(B) Alignment showing conservation of sirtuin catalytic site residues. Residues N152, D154, and H184 of Hst3 correspond respectively to N101, D103, and H118 of the Af2 sirtuin; the structure of Af2Sir2 was solved in a complex with NAD^+ and interactions with nicotinamide and nicotinamide ribose are indicated. Af2Sir2 residues corresponding to those mutated in Hst3 are colored orange; numbers in parentheses are Hst3 residues. Curved lines represent van der Waals interactions; dashed lines indicate hydrogen bonds. Adapted from [28].

(C) *hst4* null mutant strains expressing either no Hst3 or Myc-tagged Hst3 with the indicated mutations were arrested in G2/M phase with nocodazole. Immunoblots were probed for Hst3-13Myc, K56Ac, or total H3.

(D) The proportion of K56Ac H3 was quantified by mass spectrometry of histones purified from nocodazole-arrested *hst4* mutant cells expressing wild-type or Hst3 point mutants.

(E) In *hst4* null mutants, point mutations of Hst3 nicotinamide binding residues (N152A and D154A) result in a thermosensitive phenotype. 5-fold serial dilutions of each strain were plated on minimal medium and grown at either 25ºC or 37ºC in the presence of 5-FOA to select against a URA^+ plasmid encoding wild-type *HST3*.

do not eliminate catalytic activity ([30, 32]). The Hst3p missense mutants were introduced into *hst3 hst4* mutant cells. The C-pocket mutants, but not H184A, were expressed at somewhat lower levels than wild-type Hst3p (Figure 2C and data not shown), suggesting that C-pocket mutations decrease Hst3p stability. However, because Hst3p C-pocket mutations are essentially null (Figure 2E) and *hst3 hst4* mutants are defective in *CEN* plasmid maintenance [20], it is equally plausible that plasmids encoding C-pocket mutants are maintained in fewer copies per cell than those encoding wild-type or the H184A mutant Hst3p. The Hst3p N152A C-pocket mutation led to K56 hyperacetylation, comparable to *hst3 hst4* null mutants (Figure 2C). The use of a 2 μ plasmid, rather than a *CEN* plasmid, increased the expression of C-pocket mutants roughly 3-fold, but failed to decrease K56Ac (data not shown), suggesting that these mutants were completely inactive. The Hst3p H184A mutation resulted in a substantial but less pronounced increase in K56Ac (Figure 2C). These results were corroborated by quantitative mass spectrometry of histones isolated from nocodazole-arrested cells. In *hst3-N152A hst4* or *hst3-D154A hst4* mutant cells, virtually all H3 (97.7%) was K56-acetylated in G2/M, compared with 45.2% in *hst3-H184 hst4* mutants and only 24.5% in *HST3 hst4* cells (Figures 2D, Figures S3 and S4). We also examined whether other known acetylation sites in H3 and H4 were affected by mutations in Hst3/Hst4p. Based on mass spectrometry, peptides containing lysines 5, 8, 12, and 16 of histone H4 and lysines 9, 14, 18, and 23 of H3 were not significantly more acetylated in *hst3*-N152A *hst4* mutants than in wild-type cells (Figure S5). Thus, absence of Hst3 and Hst4p did not affect acetylation of any known sites, including several lysines modified in newly synthesized histones [4, 10]. As in *hst3 hst4* mutants, Hst3p C-pocket mutations conferred thermosensitivity (Figure 2E) and other phenotypes (Figure S2). In contrast, only mild phenotypes were observed in *hst3-H184A hst4* mutants (Figure 2E and Figure S2) despite significantly increased K56Ac (Figures 2C and 2D). We tested 12 additional point mutations in Hst3p phenotypically and for K56Ac abundance. In all cases, phenotypic severity correlated well with K56Ac levels (Figure S2). Thus, *hst3 hst4* mutant phenotypes occur only in cells with very high K56Ac.

hst3 hst4 Phenotypes Result from K56 Hyperacetylation

To test whether the phenotypes of *hst3 hst4* mutants resulted from a failure to remove K56Ac, we mutated lysine 56 to a nonacetylatable arginine (*hht1-K56R*). Remarkably, *hht1-K56R* rescued growth at 37ºC of *hst3 hst4* double mutants (Figure 3A). *hst3 hst4* cells are hypersensitive to genotoxic agents that interfere with replication fork progression (Figure 3A). These include hydroxyurea (HU) and methyl methane sulfonate (MMS). MMS-alkylated bases are removed by DNA glycosylases, and the resulting abasic sites are converted into nicks by apurinic endonuclease [33]. The persistence of nicks is potentially cytotoxic, particularly when converted into DNA double-strand breaks during replication [34, 35]. Hypersensitivity of *hst3 hst4* mutants to these agents was suppressed by *hht1-K56R* (Figure 3A). However, the *hst3 hst4 hht1-K56R* mutant remained considerably more sensitive to HU and MMS than wild-type cells (Figure 3A). This is consistent with the fact that *hht1-K56R* itself confers sensitivity to HU and MMS (Figure 3A) [5–7, 9]. Hence, both failure to acetylate and failure to deacetylate H3 K56 sensitize cells to HU/MMS, albeit to different degrees. A far more acute sensitivity to these genotoxic agents is observed when K56 is globally acetylated (*hst3 hst4*) than in mutants in which K56 acetylation is not possible (*hht1-K56R*, Figure 3A). Remarkably, suppression of the HU/MMS sensitivity of *hst3 hst4* double mutants by the *hht1-K56R* mutation was more complete in cells that coexpressed wild-type H3 (compare Figures 3B and 3A), presumably because moderate K56Ac occurred in wild-type H3. Thus, both K56Ac and its controlled deacetylation by Hst3/Hst4p are required for an optimal response to genotoxic agents. These results are not inconsistent with previously published data showing that K56Ac deacetylation is delayed by the DNA damage checkpoint in response to DNA breaks during replication [6]. In wild-type cells, the retention of K56Ac triggered by DNA damage is only transient and K56Ac is eventually removed after DNA repair in G2 [6]. In contrast, K56Ac is permanent and genome wide in *hst3 hst4* mutants. Therefore, although H3 K56R and *hst3 hst4* mutants are both sensitive to genotoxic agents that interfere with replication, the underlying sources of sensitivity are likely different.

Given the genome-wide nature of H3 K56Ac, it seemed likely that failure to deacetylate in G2 would interfere with mitosis. To address this, the mitotic chromosome loss phenotype of *hst3 hst4* mutants [20] was evaluated. For this purpose, a chromosome fragment was generated in *hst3 hst4 hht1-K56R hht2-hhf2* heterozygous diploid cells. After sporulation, haploid strains with the desired genotypes that carry the chromosome fragment were assayed for colony sectoring [36]. In this assay, colonies that retain the chromosome fragment are white and colonies without it turn red. Frequent mitotic chromosome-loss events generate colonies with red and white sectors. By itself, the *hht1-K56R* mutation did not produce sectored colonies (data not shown). As reported previously, *hst3 hst4* colonies sector very frequently (Figure 3C). Colony sectoring was substantially suppressed in *hst3 hst4 hht1-K56R* mutant cells relative to *hst3 hst4* mutants. Thus, the high-frequency mitotic chromosome loss in *hst3 hst4* double mutants also stems from K56 hyperacetylation.

The Histone Chaperone Asf1p Is Needed for H3 K56 Acetylation

Given that K56Ac occurs in newly synthesized H3, it seemed likely that mutations of histone chaperones that promote deposition of K56Ac H3 into chromatin would also suppress *hst3 hst4* phenotypes. Asf1p is a chaperone that functions in two distinct pathways of histone deposition mediated by either Hir proteins or chromatin assembly factor 1 [37, 38]. Interestingly, a null mutation in *asf1* strongly suppressed the Ts^- (Figure 4A) and other phenotypes (data not shown) of *hst3 hst4* mutants. Since H3 mutations that abolish K56Ac also suppress *hst3 hst4* mutants, this prompted us to investigate whether *asf1* mutants had defects in K56Ac. As predicted, K56Ac was undetectable in *asf1* mutants, both in normally proliferating cells and in HU-treated

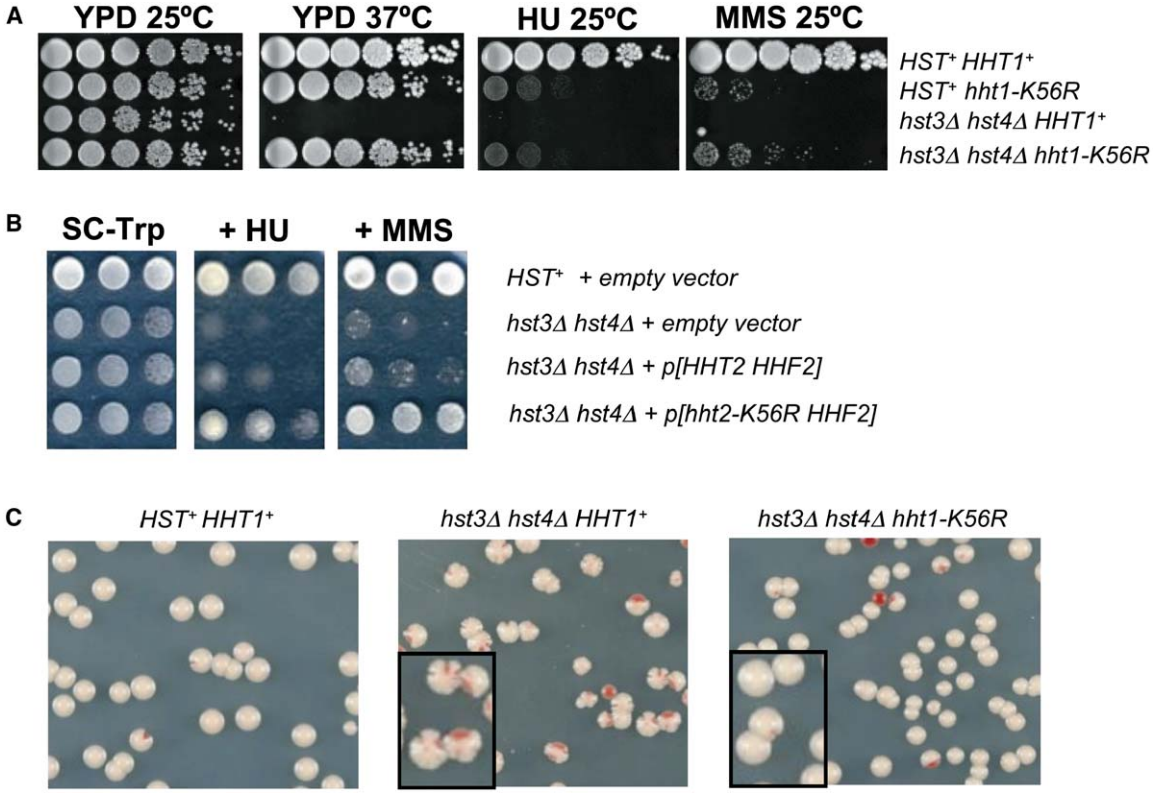

Figure 3. The Phenotypes of *hst3 hst4* Mutants Are Suppressed by Mutation of Histone H3 K56 to Arginine

(A) The thermosensitivity and genotoxic agent sensitivity of *hst3 hst4* mutants are suppressed by the *hht1-K56R* mutation. 5-fold serial dilutions of each strain were plated on rich medium and grown at 25ºC or 37ºC for 4 days. For drug sensitivity, medium contained either 0.1 M HU or 0.01% MMS and growth was for 6 days. Relevant genotypes are shown; cells carried deletions in *hht2-hhf2*.

(B) The *hht2-K56R* mutation suppresses the HU and MMS sensitivity of *hst3 hst4* cells that coexpress wild-type H3. *CEN* plasmids encoding wild-type H3 or H3 K56R were transformed into *hst3 hst4* mutants. 5-fold serial dilutions of each strain were grown at 25ºC for 6 days on medium lacking tryptophan (to select for plasmids) and containing compounds as in (A).

(C) The *hht1-K56R* mutation suppresses the mitotic chromosome-loss phenotype of *hst3 hst4* mutants. Haploid cells harboring a nonessential chromosome fragment (chr *VII RAD2d*) carrying a *SUP11°* allele that suppresses *ade2-1* were grown in minimal medium with limited adenine for 5 days. Cells that retain the chromosome fragment are white, whereas cells that lose the fragment are red. *HST+ hht1-K56R* control colonies (not shown) were indistinguishable from *HST+ HHT1+* colonies. Colony sectoring indicates a high frequency of mitotic chromosome loss. The differences between *hst3 hst4* and *hst3 hst4 hht1-K56R* cells were observed with five independent isolates of each genotype. All strains carried an *hht2-hhf2* deletion, which had no effect on phenotype.

cells (Figure 4B). This important result was reported elsewhere after this paper was submitted [9] and argues that DNA damage sensitivity in *asf1* mutants results from a lack of K56Ac. Two models could explain the fact that Asf1p is needed for K56Ac. One model proposes that Asf1p is needed for recognition of newly synthesized H3 by HATs that modify K56; another suggests that Asf1p binding protects H3 from premature deacetylation. The latter model predicts that K56Ac should be present in cells lacking both Asf1p and the activities that deacetylate K56. However, as expected from the fact that the *asf1* mutation suppressed the phenotypes of *hst3 hst4* mutants, H3 K56Ac was undetectable in *hst3 hst4 asf1* triple mutants (Figure 4C), arguing that Asf1p functions to promote K56Ac of newly synthesized H3.

Replication-Induced Lesions and Chronic DNA Damage Checkpoint Activation in *hst3 hst4* Mutants

Transcriptional profiling of *hst3 hst4* mutants identified *RNR3* and *HUG1* as highly upregulated transcripts. These transcripts are induced by genotoxic agents that interfere with DNA replication [39, 40]. They were upregulated by >20-fold in *hst3 hst4* mutant cells, indicating that *hst3 hst4* mutants experience significant levels of spontaneous DNA damage. To confirm this, we monitored canonical markers of DNA damage and checkpoint activation, namely phosphorylation of histone H2A S128 (γ-H2A) and Rad53p [41]. γ-H2A increased during normal progression through S phase even in wild-type cells, most likely as a result of DNA double-strand breaks (DSBs) generated during normal replication [42]. However, the S phase γ-H2A levels were substantially elevated in *hst3 hst4* mutants (Figure 5). In wild-type cells, the damage that triggered γ-H2A during S phase was transient (Figure 5). In contrast, even at the permissive temperature, γ-H2A persisted well into G2 in *hst3 hst4* mutants. Rad53 hyperphosphorylation was also observed in *hst3 hst4* mutants and was suppressed by *hht1-K56R*, implying that persistence of replication-induced DNA damage in *hst3 hst4* mutants resulted from K56Ac hyperacetylation (I.C., unpublished data).

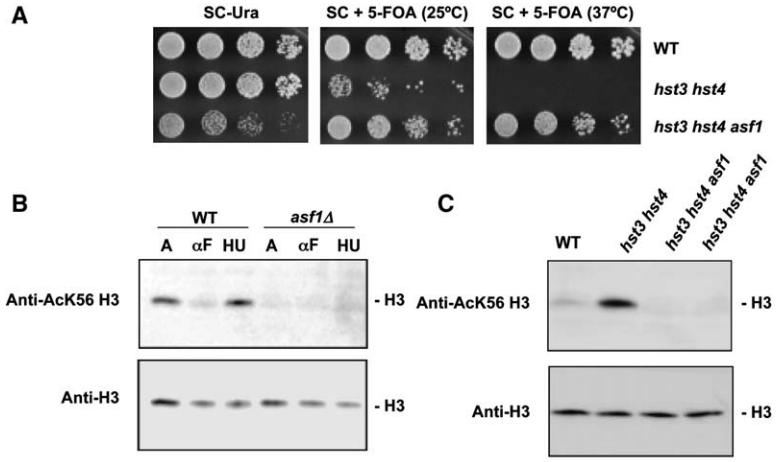

Figure 4. The Asf1p Histone Chaperone Is Needed for H3 K56Ac

(A) The *asf1* mutation suppresses the thermosensitive phenotype of *hst3 hst4* mutants. 5-fold serial dilutions of the indicated strains containing the *HST3*$^+$ gene on a *URA3* plasmid were grown at 25ºC or 37ºC on media containing 5-FOA and uracil.

(B) Whole-cell lysates were prepared from asynchronous (A), G1-arrested (αF), or hydroxyurea-treated (HU) cells. Immunoblots were probed for K56Ac or total H3.

(C) Immunoblots of the indicated strains were analyzed for K56Ac or total H3.

Thus, *hst3 hst4* mutant cells either sustained more damage during replication or repaired DNA replication-induced damage less efficiently than wild-type cells. To test these possibilities, we determined whether perturbations of the replisome, the DNA damage checkpoint, or DSB repair were detrimental to *hst3 hst4* mutants. Mutations that cripple DNA replication, the DNA damage checkpoint, or DSB repair by homologous recombination were all synthetic lethal with *hst3 hst4*, but not with single *hst* mutations. Like other *hst3 hst4* phenotypes, these lethalities were partially suppressed by the *hht1-K56R* allele. Remarkably, two tagged alleles of essential DNA replication genes, *CDC45* (encoding a protein that functions with the MCM DNA helicase) and *POL30* (encoding PCNA), both of which support normal growth in the absence of a wild-type allele, are also synthetic lethal with *hst3 hst4* (I.C., unpublished data). Thus, even subtle perturbations in DNA replication are cytotoxic to cells with K56 hyperacetylation. These results suggest that both the DNA damage checkpoint and homologous recombination function properly in *hst3 hst4* mutants and are critical for viability in the face of K56 hyperacetylation. Studies of HU- and MMS-treated cells also showed a normal checkpoint response in *hst3 hst4* mutant cells (I.C., unpublished data). Together with earlier studies [43, 44], this suggests an important role for Hst3 and Hst4p in preventing the generation of lethal DNA lesions during replication.

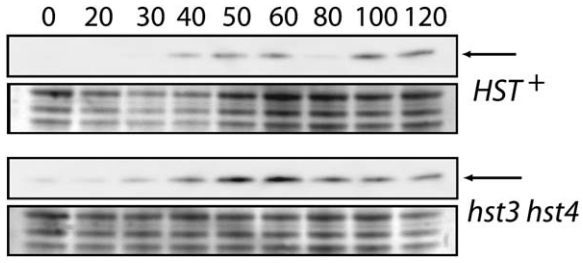

Figure 5. Chronic γ-H2A Phosphorylation in *hst3 hst4* Mutants

Cells were arrested with α-factor and released into YPD medium at 25ºC. Samples were taken at the indicated times (min), and immunoblots were probed for γ-H2A or stained with Ponceau as loading control (bottom panels). Note the persistence of γ-H2A signals (arrows) throughout the cell cycle in the *hst3 hst4* mutant.

In order to investigate the source of these phenotypes, we created a yeast strain with a "rapidly inactivatable" allele of *HST3*. In this *hst4Δ* strain, *HST3* was fused to a temperature-inducible degron [45] expressed from a methionine-repressible promoter (*td-HST3*). The *td-HST3 hst4Δ* strain grew normally under permissive conditions (no methionine, 25ºC) but was temperature sensitive in 5 mM methionine at 37ºC (Figure 6A). To monitor degradation, td-Hst3p was tagged with Myc_{13}. Upon addition of methionine, the transcription of *td-HST3* was repressed and a simultaneous shift to elevated temperature triggered rapid degradation of residual td-Hst3p (Figure 6B). Consistent with the destruction of td-Hst3p (and absence of Hst4p), we observed a rapid increase in K56Ac detectable at the earliest time point after td-Hst3p inactivation (Figure 6B, 15 min).

The conditional allele *td-Hst3-13Myc* enabled us to determine how soon S phase-related phenotypes occurred after td-Hst3p degradation. For this purpose, we examined the effect(s) of depleting Hst3p in synchronized cells. *HST3* and *td-HST3 hst4Δ* cells were arrested in α-factor at the permissive temperature, then released into the cell cycle under restrictive conditions. In *td-HST3 hst4Δ* cells, Hst3p-Myc_{13} disappeared rapidly and was undetectable by the onset of the first S phase. K56Ac levels increased during S phase, but only to levels observed in wild-type cells (Figure 6C). Likewise, γ-H2A was detectable during the first cell cycle in the absence of Hst3p/Hst4p, but only at wild-type levels (Figure 6C, 0–90 min). There was no apparent delay in cell-cycle progression as judged by FACS (Figure S7). However, differences between *td-HST3 hst4* mutants and wild-type cells emerged after this first S phase. First, in the absence of Hst3/Hst4p, K56Ac did not disappear in G2 (Figure 6C, compare 60–105 min lanes). Second, as cells entered the subsequent S phase, γ-H2A rose to higher levels in Hst3p-depleted cells than in wild-type cells (Figure 6C, 135–150 min), presumably due to K56 hyperacetylation.

The absence of phenotype during the first S phase was not due to incomplete inactivation of td-Hst3p; 50% of H3 isolated after one round of replication under these restrictive conditions was K56 acetylated (Figure S6). Given that K56Ac is restricted to newly synthesized H3 [6], 50% acetylation is the theoretical limit

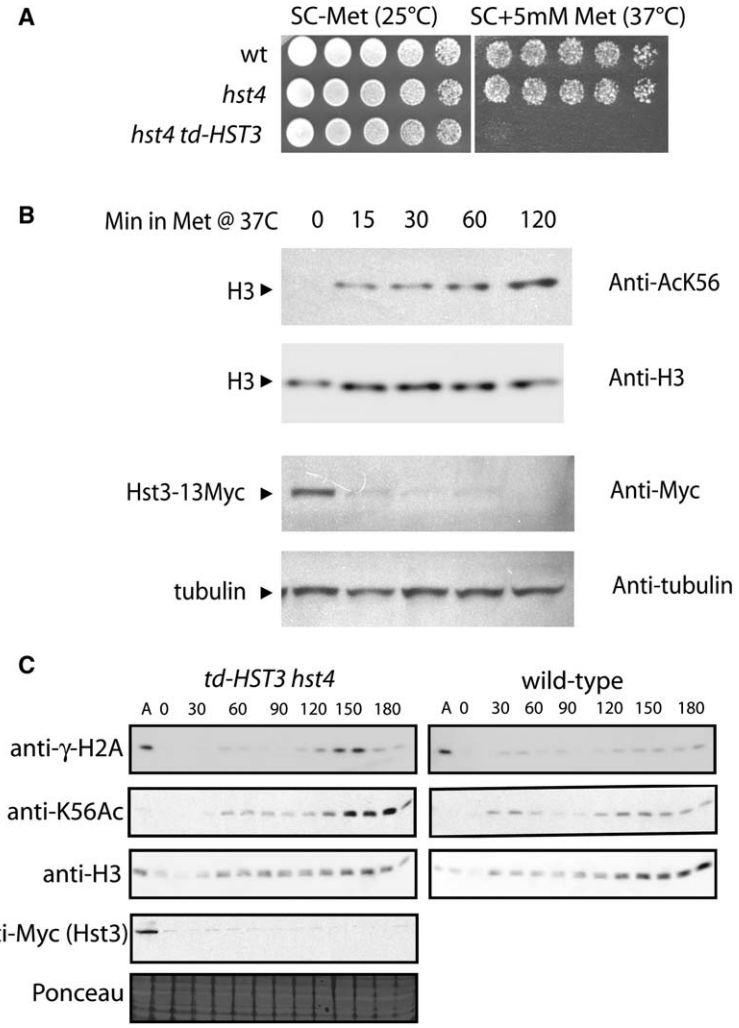

Figure 6. Rapid Inactivation of Hst3p Triggers Accumulation of K56Ac and γ-H2A

(A) Phenotypes of *td-HST3 hst4* cells. Like *hst3 hst4* null mutants, *td-HST3 hst4* cells are temperature sensitive.

(B) Upon shift to nonpermissive conditions, td-Hst3-13MYC is rapidly degraded and a concomitant increase in K56Ac is observed. An asynchronous culture of td-*HST3 hst4* cells grown under permissive conditions was shifted to nonpermissive conditions (5 mM methionine at 37°C) and sampled at the indicated times. Immunoblots were performed to detect td-Hst3-13Myc, K56Ac, and total H3.

(C) Hst3p depletion causes K56Ac and γ-H2A during S phase. α-factor arrested cultures of td-*HST3 hst4* and control (wild-type) cells grown under permissive conditions were simultaneously released from G1 arrest, shifted to nonpermissive conditions, and sampled at the indicated times (min). FACS profiles after Hst3p depletion were normal (Figure S7).

expected if all newly synthesized H3 molecules deposited genome-wide are K56 acetylated and deacetylation is completely abolished in *td-HST3 hst4Δ* mutant cells. In support of this, the same percentage of K56Ac was observed when wild-type cells were treated with nicotinamide just before S phase, and K56Ac was analyzed in G2 (Figure S6). The fact that K56Ac never rises significantly above 50% when Hst3/Hst4p are inhibited (Figure S6) strongly argues that parental histones do not undergo Hst3/Hst4-dependent rapid turnover of K56Ac during S phase. These experiments suggest that parental histones are not K56 acetylated in front of the replication apparatus and then deacetylated after transfer behind the replisome. We conclude that K56Ac in normal cells occurs in essentially all newly synthesized H3, but not in parental H3.

Hst3p Can Trigger K56Ac Deacetylation in Mature Chromatin

Normally, K56Ac peaks during late S and vanishes in G2/M [6, 10]. In contrast, *HST3* mRNA and Hst3p are present in low abundance during S phase but increase substantially during G2/M ([25]; data not shown). These reciprocal patterns of K56Ac and Hst3p suggest that most K56Ac is normally deacetylated in the context of mature chromatin during G2. To test this, a culture of *td-HST3 hst4Δ* cells was grown under restrictive conditions to generate fully acetylated chromatin and arrested in G1 with α-factor or in G2/M with nocodazole. Cells were shifted to permissive conditions while maintaining the G1 or G2/M arrest. Under these conditions, td-Hst3p induction led to rapid deacetylation of K56Ac in the arrested cells (Figure 7). This experiment ruled out the formal possibility that Hst3p functions as a negative regulator of the HATs that modify K56. Since histone genes are repressed in G1 and G2, essentially all K56Ac H3 molecules in these cells were packaged into chromatin before Hst3p induction. Thus, Hst3p can trigger deacetylation of mature chromatin. Because K56Ac is important in response to DNA damage during S phase [5, 6], this result implies that cells must carefully control the expression and/or deacetylase activity of Hst3p in G1 and S to ensure that nascent chromatin is not prematurely deacetylated during replication. Remarkably, even when maintained under permissive conditions for expression, td-Hst3p is eventually degraded in parallel with the deacetylation of K56 in G1- or G2/M-arrested cells (Figure 7). This result suggests that Hst3p stability is feedback regulated by K56Ac levels (see Discussion).

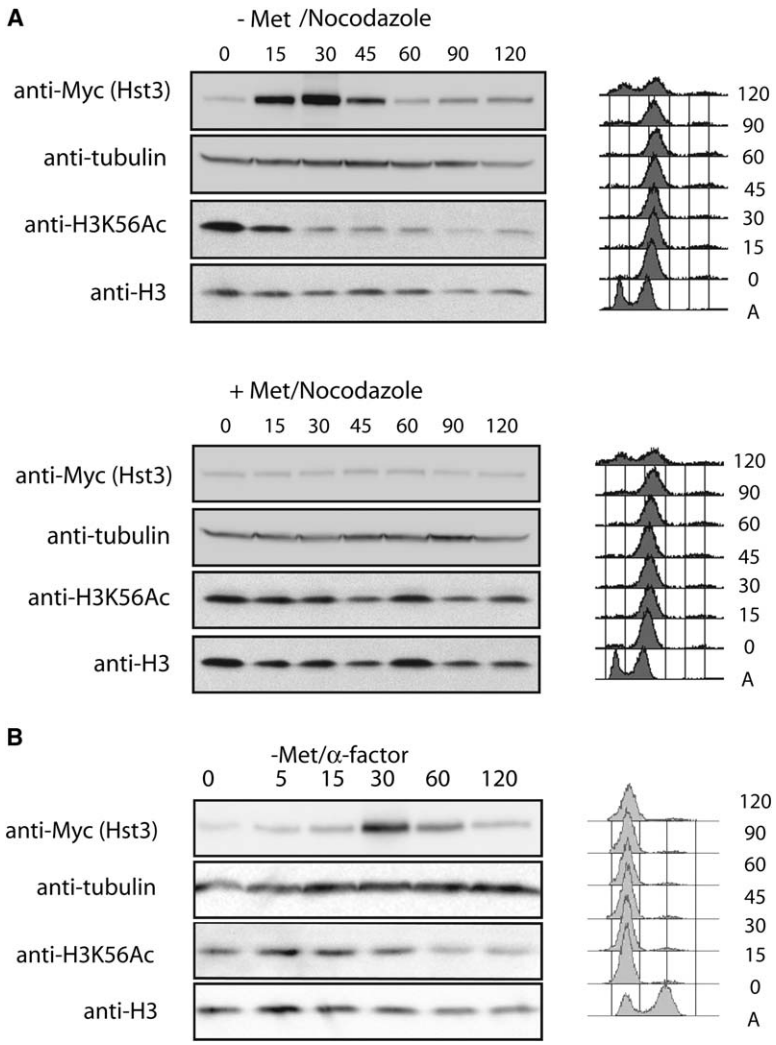

Figure 7. Induction of Hst3p Synthesis Triggers H3 K56 Deacetylation in Mature Chromatin

(A) td-*HST3 hst4* cells were grown under nonpermissive conditions and arrested in G2/M phase with nocodazole. To induce td-Hst3p, the culture was shifted to permissive conditions and sampled at the indicated times. Note that Hst3p transiently accumulates to a high level after 30 min, and a significant drop in K56Ac signal is observed at the same time point. Cells kept under nonpermissive conditions for the whole experiment served as a negative control.

(B) td-*HST3 hst4* cells grown under nonpermissive conditions were arrested in G1 with α-factor and treated as in (A).

Discussion

Cell-Cycle Control of Hst3/Hst4p and K56 Deacetylation

Hst3p and Hst4p are closely related and genetically redundant proteins. Consistent with this, only *hst3 hst4* double mutants, and not single mutants, have virtually 100% K56-acetylated H3 throughout the cell cycle (Figure 1). To our knowledge, such massive hyperacetylation of a single residue is unprecedented. Both K56 hyperacetylation and lack thereof have profound impacts on cellular resistance to genotoxic agents (Figure 3). Thus, both acetylation and deacetylation must be carefully regulated in proliferating cells. Since K56 acetylation occurs only on newly synthesized histones, most likely before deposition into chromatin, part of this regulation is achieved through cell-cycle control of histone synthesis. Substrate availability may be the main mechanism regulating K56 acetylation timing, since H3 expressed outside S phase can be acetylated [6], implying that K56 acetyltransferase(s) are constitutive. Hence, the timing of expression and/or activation of the K56Ac deacetylases are critical for maintaining genome integrity. *HST3* and *HST4* mRNAs are cell-cycle regulated, peaking in G2/M and late M, respectively [25]. Additionally, our results raise the exciting possibility that Hst3p is also controlled at the level of protein stability, possibly in response to changes in K56Ac levels. Even when ectopically expressed, td-Hst3p is rapidly degraded in parallel with K56Ac deacetylation in G2/M and G1 (Figure 7), suggesting that K56Ac deacetylation signals td-Hst3p degradation. Interestingly, native Hst3p is also degraded during G2/M (data not shown). Such feedback regulation would ensure the absence of Hst3p in G1, providing a window of opportunity for K56Ac accumulation in nascent chromatin and execution of its genome-protective role during replication. Rising K56Ac levels during late S phase [6, 10] would then stabilize Hst3p, as a necessary prelude for efficient removal of K56Ac in G2/M. Global deacetylation of K56Ac in G2/M probably serves two important functions. First, it prevents mitotic chromosome loss caused by K56 hyperacetylation (Figure 3C). Second, it precludes the presence of K56Ac ahead of replication forks, a condition that may promote spontaneous damage during replication (Figures 5 and 6C).

Are Hst3p and Hst4p Directly Responsible for K56 Deacetylation?

We have not reconstituted Hst3/Hst4p-dependent deacetylation of K56Ac in vitro, but our data support the

notion that Hst3p and Hst4p are K56Ac deacetylases. (1) Nicotinamide and point mutations that should cripple NAD^+ binding to Hst3p inhibit K56Ac deacetylation. (2) No other candidate deacetylase exists because all known deacetylases have been knocked out, individually and in certain combinations, without affecting K56Ac deacetylation (Figure 1 and data not shown). (3) The fast kinetics of K56Ac accumulation in the *td-HST3* strain upon a shift to nonpermissive conditions is consistent with direct deacetylation by Hst3p. (4) Most importantly, de novo expression of Hst3p in G1- or G2/M-arrested cells triggers a rapid loss of pre-existing K56Ac. This implies that Hst3p acts by promoting K56Ac deacetylation in mature chromatin, rather than by negatively controlling K56 HATs.

K56Ac reaches 50% after a single round of DNA replication in the absence of Hst3/Hst4p, strongly implying that most newly synthesized H3 molecules are K56 acetylated when deposited during nucleosome assembly. However, Hst3p expression peaks during G2/M, rather than S phase ([25]; data not shown). This suggests that there is little K56Ac turnover during S phase. Hence, robust Hst3p expression in G2/M is probably critical for complete deacetylation. In the crystal structure of the nucleosome, several residues adjacent to K56 contribute H3/DNA contacts both at the dyad axis and near the DNA entry and exit points [46]. These contacts could sterically impede access to Hst3p and interfere with K56Ac deacetylation in mature chromatin. Indeed, the kinetics of K56Ac deacetylation in vivo is significantly slower than that of N-terminal tail lysines [14]. Thus, K56Ac deacetylation in mature chromatin probably requires the concerted action of both Hst3p and other proteins that distort histone-DNA contacts to expose K56Ac to Hst3p.

Our experiments argue that the pleiotropic effects of *hst3 hst4* mutations directly result from K56 hyperacetylation. Every phenotype tested, including mitotic chromosome loss, is suppressed by expression of the K56R mutant. This suppression occurs even when H3 K56R is coexpressed with wild-type H3, suggesting that chromatin consisting of both acetylated and nonacetylated nucleosomes, or perhaps even mixed nucleosomes, does not result in the major phenotypes seen with hyperacetylated chromatin. Consistent with this, the *hst3-H184A* mutation results in 45% K56Ac, but has very mild phenotypes relative to Hst3 mutations leading to 98% acetylation (Figures 2D and 2E, Figure S2). The *hst3 hst4* mutants suffer from spontaneous damage based on sensitive markers of DNA damage (Figure 5) and synthetic lethality with mutations that cripple the DNA damage checkpoint and homologous recombination-mediated repair. One important unresolved issue is the exact source of the spontaneous damage experienced by proliferating cells lacking Hst3/Hst4p. When td-Hst3p is inactivated, we observe elevated γ-H2A during S phase, suggesting a role for Hst3p in preventing replication fork stalling/collapse. Interestingly, this is only detectable in the second S phase after td-Hst3p depletion, even though turnover of K56Ac in nascent chromatin is completely blocked during the first cell cycle. One possibility (Figure S8, Model 1) is that spontaneous damage during replication happens only after K56Ac has reached a critical level substantially exceeding 50%, which requires more than a single round of replication in the absence of Hst3/Hst4p. Alternatively (Figure S8, Model 2), this type of damage might occur far more frequently when replisomes traverse "abnormal" chromatin segments in which parental nucleosomes are K56 acetylated. The damage caused by K56 hyperacetylation could occur stochastically throughout the genome or may be restricted to specific regions that are intrinsically difficult to replicate. In addition, given that K56Ac is normally restricted to replicated DNA regions in wild-type cells (Figure S8), the abnormal presence of K56Ac both in front and behind replication forks may cause rereplication of specific DNA segments in *hst3 hst4* mutants. Rereplication is known to trigger DNA damage and checkpoint activation in yeast [47]. In summary, it is remarkable that both the acetylation and the deacetylation of a single lysine residue in histone H3 have such profound effects on several aspects of chromatin physiology. Many other lysines are transiently acetylated in newly synthesized histones [3, 4, 10, 11]. This raises the fascinating possibility that regulated turnover of other acetylated lysines may have evolved unique functions in the maintenance of genome integrity.

Experimental Procedures

These are described in the Supplemental Data.

Supplemental Data

Supplemental Data include eight figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/13/1280/DC1/.

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